FORGETTER2 protein phosphatase and phospholipase D modulate heat stress memory in Arabidopsis

Urrea Castellanos, Reynel; Friedrich, Thomas; Petrovic, Nevena; Altmann, Simone; Brzezinka, Krzysztof; Gorka, Michal

Published in:
Plant Journal

DOI:
10.1111/tpj.14927

Publication date:
2020

the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2020) doi: 10.1111/tpj.14927

FORGETTER2 protein phosphatase and phospholipase D modulate heat stress memory in Arabidopsis

Reynel Urrea Castellanos[1,†], Thomas Friedrich[1], Nevena Petrovic[1], Simone Altmann[1,‡], Krzysztof Brzezinka[1], Michal Gorka[2], Alexander Graf[2] and Isabel Bäurle[1,*]

[1]*Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Strasse 24-25, Potsdam 14476, Germany,*
[2]*Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, Potsdam 14476, Germany*

Received 18 March 2020; revised 12 June 2020; accepted 1 July 2020.
*For correspondence (e-mail isabel.baeurle@uni-potsdam.de).
[†]Present address: Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, Potsdam 14476, Germany
[‡]Present address: School of Life Sciences, University of Dundee, Dundee, DD2 5DA, UK

SUMMARY

Plants can mitigate environmental stress conditions through acclimation. In the case of fluctuating stress conditions such as high temperatures, maintaining a stress memory enables a more efficient response upon recurring stress. In a genetic screen for *Arabidopsis thaliana* mutants impaired in the memory of heat stress (HS) we have isolated the *FORGETTER2* (*FGT2*) gene, which encodes a type 2C protein phosphatase (PP2C) of the D-clade. *Fgt2* mutants acquire thermotolerance normally; however, they are defective in the memory of HS. FGT2 interacts with phospholipase D α2 (PLDα2), which is involved in the metabolism of membrane phospholipids and is also required for HS memory. In summary, we have uncovered a previously unknown component of HS memory and identified the FGT2 protein phosphatase and PLDα2 as crucial players, suggesting that phosphatidic acid-dependent signaling or membrane composition dynamics underlie HS memory.

Keywords: priming, protein phosphatase, stress memory, heat stress, *Arabidopsis thaliana*.

INTRODUCTION

Rising mean temperatures and temperature extremes are predicted to cause severe yield losses in major crops during the coming decades (Battisti and Naylor, 2009; Lobell *et al.*, 2011). Thus, improving heat tolerance is a major breeding target. The immediate responses to heat stress (HS) are well studied. However, in nature HS is often chronic or recurring, suggesting that temporal dynamics of HS responses are highly relevant, but under-researched. Recent findings demonstrated that after exposure to HS plants retain an active memory of HS that allows them to withstand a recurring HS better, compared to an unprimed plant (Charng *et al.*, 2006; Charng *et al.*, 2007; Stief *et al.*, 2014; Bäurle, 2016; Brzezinka *et al.*, 2016; Lämke *et al.*, 2016). Such priming has also been observed in response to other biotic and abiotic stress cues, suggesting that this is an important component of plant stress responses (Jaskiewicz *et al.*, 2011; Ding *et al.*, 2012; Sani *et al.*, 2013; Singh *et al.*, 2014; Hilker *et al.*, 2016; Feng *et al.*, 2016; Lämke and Bäurle, 2017). In extreme cases, such a stress memory may be stable over multiple growth seasons or even inherited to the next generation, as has been shown for hyperosmotic stress, pathogen attack, and herbivory (Luna *et al.*, 2012; Rasmann *et al.*, 2012; Wibowo *et al.*, 2016; Lämke and Bäurle, 2017).

The transcriptional response to HS is mediated by a subset of the 21 *HEAT SHOCK FACTOR* (*HSF*) genes in *Arabidopsis thaliana* (Scharf *et al.*, 2012; Ohama *et al.*, 2017). So far, eight HSFs have been implicated in the acquisition of thermotolerance, with among them the four isoforms of HSFA1, A1A, A1B, A1D, and A1E, which are considered as master regulators of the immediate HS responses (Mishra *et al.*, 2002; Schramm *et al.*, 2008; Liu *et al.*, 2011; Nishizawa-Yokoi *et al.*, 2011; Yoshida *et al.*, 2011; Mittler *et al.*, 2012; Yeh *et al.*, 2012; Liu and Charng, 2013). They are post-translationally activated upon HS and induce the transcription of a suite of target genes, including many heat shock proteins (HSPs) that act as chaperones and restore proteostasis. They also induce the expression of *HSFA2*, which is the only known HSF that regulates HS memory (maintenance of acquired thermotolerance) and which in turn amplifies the transcriptional induction of a subset of HS response genes (Schramm *et al.*, 2006; Charng *et al.*, 2007; Liu *et al.*, 2011; Nishizawa-Yokoi *et al.*, 2011). This

subset overlaps with the genes that have been classified as HS memory genes because of their sustained induced expression after HS that lasts for at least 2 days (Stief *et al.*, 2014). One of these genes with a sustained induction after HS is *HEAT SHOCK-ASSOCIATED PROTEIN 32* (*HSA32*) (Charng *et al.*, 2006; Brzezinka *et al.*, 2016). Like *HSFA2*, *HSA32* is essential for HS memory, but dispensable for the acquisition of thermotolerance and basal thermotolerance (Charng *et al.*, 2006). In addition, HSFA2 binds transiently at HS memory genes, which leads to sustained chromatin modifications, such as histone H3K4 dimethylation and trimethylation, that are associated with sustained induction (Lämke *et al.*, 2016). The *FORGETTER1* gene mediates low nucleosome occupancy to sustain gene induction during HS memory (Brzezinka *et al.*, 2016). Besides this strong evidence for a chromatin basis of HS memory, protein stabilization through protein–protein interactions as well as regulated protein degradation has also been implicated in HS memory (Wu *et al.*, 2013; Sedaghatmehr *et al.*, 2016), indicating that other cellular components contribute to HS memory.

The mechanisms underlying initial sensing of HS and the activation of the HS response remain poorly understood (Ohama *et al.*, 2017). Changes in membrane fluidity and subsequent activation of Ca^{2+}-channels are very rapid, and hence it has been proposed that this triggers a Ca^{2+}-dependent phosphorylation cascade resulting in HSFA1 activation (Zhang *et al.*, 2009; Saidi *et al.*, 2009). HS also results in rapid accumulation of reactive oxygen species (ROS) in the chloroplast, and this may trigger a parallel pathway of HSFA1 activation (Volkov *et al.*, 2006; Konigshofer *et al.*, 2008). In any case, a model where HSFs are activated by releasing bound chaperones towards denatured proteins is insufficient to explain HS sensing (Ohama *et al.*, 2017).

Here, we have identified the FORGETTER2 (FGT2) protein phosphatase as a novel regulator of HS memory in *A. thaliana* through forward genetic screening. FGT2 is a type 2C protein phosphatase (Fuchs *et al.*, 2013), and it is localized at the plasma membrane (PM). FGT2 interacts with phospholipase D α2 (PLDα2). Phospholipids and phosphatidic acid (PA), the product of PLD-mediated hydrolysis, are regulated by various stresses and act in stress signaling and acclimation (Bargmann and Munnik, 2006; Moellering and Benning, 2011; Hong *et al.*, 2016). Together, our results suggest that FGT2 and PLDα2 function in HS memory, likely by affecting lipid signaling or membrane lipid dynamics.

RESULTS

The *forgetter2-1* mutant displays a specific defect in HS memory

To identify factors specifically required for HS memory, we performed a mutagenesis screen for modifiers of the expression of the HS memory gene *HSA32* (Brzezinka *et al.*, 2016). We identified *forgetter2-1* (*fgt2-1*) as a recessive mutant with normal HS induction of *pHSA32::HSA32-LUCIFERASE* (LUC) expression, but reduced maintenance (Figure 1a). No morphological defects were apparent in *fgt2-1* mutants under normal growth conditions. To test whether the lower LUC signal correlated with reduced HS memory, 4 day-old seedlings were treated with an acclimating HS (ACC, for details see the Experimental procedures section), and 2 or 3 days later again with a tester HS (44°C) that is lethal to a plant that has not received the ACC treatment (Stief *et al.*, 2014). Indeed, *fgt2-1* displayed reduced growth and survival compared to the parental line under these conditions (Figures 1b,c and S1a). In contrast, we did not observe a defect (but rather a slight enhancement) in the acquisition of thermotolerance (both HSs applied directly after each other, with extended time at 44°C) or in the basal thermotolerance (HS at 44°C without prior ACC) (Figure S1b,c). In summary, our results indicate that the immediate HS responses in *fgt2-1* are normal and that the mutant is specifically defective in HS memory.

FGT2 encodes a protein phosphatase

To identify the mutation underlying the *fgt2-1* phenotype, recombination breakpoint mapping and genome resequencing were combined to identify a single nucleotide polymorphism (SNP) in exon 1 of *At5g66080* as the candidate mutation accounting for the *fgt2-1* phenotype (Figure 2a). The SNP causes an amino acid exchange from leucine to phenylalanine at position 44 of the protein. *At5g66080* encodes APD9/P2C79, a protein phosphatase of the type 2C family (PP2C), which are also known as metallo-dependent protein phosphatases (Schweighofer *et al.*, 2004; Fuchs *et al.*, 2013). In *A. thaliana*, the monomeric PP2C family has 80 members and is subdivided into 13 clades (Xue *et al.*, 2008). FGT2 falls into clade D together with eight other proteins (Figure S2a) (Schweighofer *et al.*, 2004; Xue *et al.*, 2008). PP2C phosphatases are involved in diverse plant processes, with clade A PP2Cs mediating ABA-dependent stress responses (Schweighofer *et al.*, 2004; Fuchs *et al.*, 2013). Previously, serine/threonine phosphatase activity was demonstrated for FGT2 *in vitro* (Takahashi *et al.*, 2012). Leucine-44 resides at the N-terminus of the PP2C catalytic domain (Smart domain SM00332, amino acid [aa] 39–354) and is widely conserved among putative FGT2 orthologs from other dicotyledonous species (Figure S2a,b), suggesting that leucine-44 may be critical for protein function. To provide additional evidence that *FGT2* is APD9, the physiological HS memory defect was confirmed in two additional insertion mutant alleles (Figure S3a,b). As in *fgt2-1*, no deficiency in the acquired or basal thermotolerance was observed in the recessive *fgt2-2* mutant (Figure S3c,d). We further confirmed the identity of *FGT2* by transgenic complementation of *fgt2-1* with

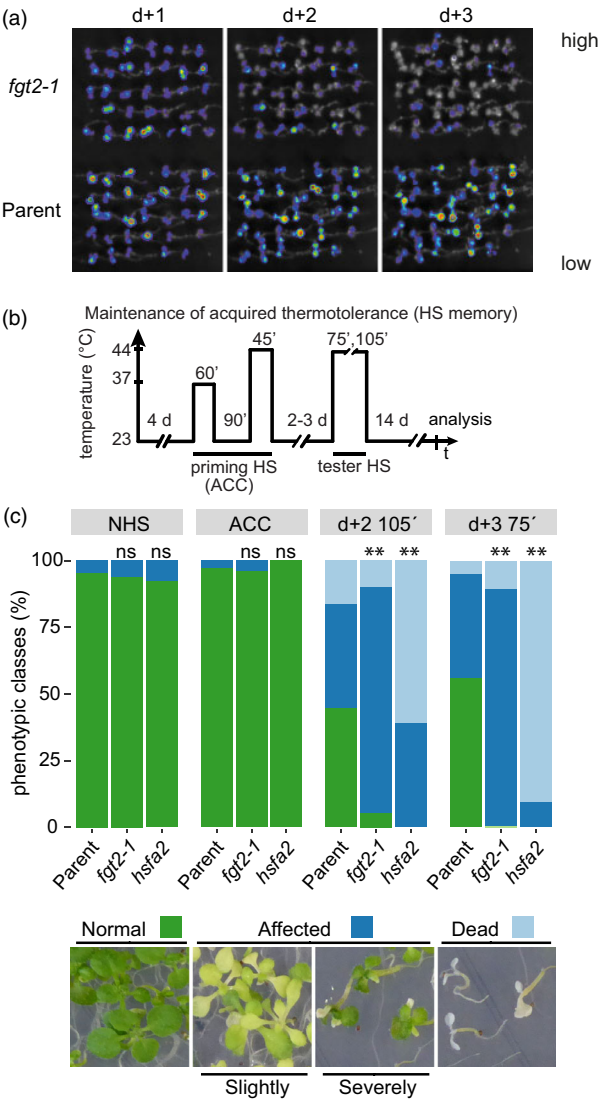

Figure 1. The *forgetter2-1* mutant (*fgt2-1*) displays a specific defect in HS memory.
(a) Bioluminescence of *pHSA32::HSA32-LUC* in *fgt2-1* and the parent assayed 1 (d+1), 2 (d+2), and 3 (d+3) days after an acclimating HS (ACC). *Fgt2-1* displays normal induction but reduced maintenance of LUC activity. The false color scale of relative LUC activity is shown. (b,c) *Fgt2-1* is impaired in HS memory at the physiological level. Four-day-old seedlings of the indicated genotypes were subjected to ACC treatment; (b) 2 or 3 days later they were exposed to a tester HS at 44°C for the indicated times, and (c) photographs were taken 14 days after ACC (Figure S1a) and seedling survival was quantified. $^{**}P < 0.001$, Fisher's exact test. NHS, no HS control. Data shown are averages over at least two independent biological replicates with each three technical replicates (84 individuals per genotype). Bottom: Representative images of the phenotypic categories used for quantifying seedling survival. Seedlings were classified into categories according to the degree of damage and survival.

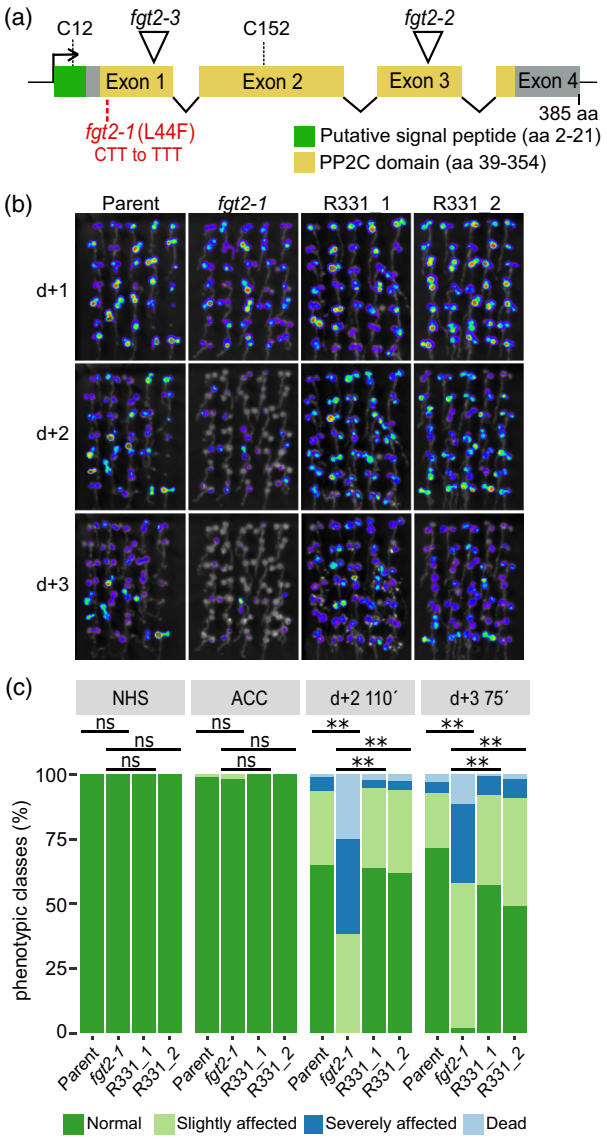

Figure 2. *FGT2* encodes a protein phosphatase of the PP2C family.
(a) Gene model of *FGT2* (*At5g66080*). Predicted domains of FGT2 protein (385 aa) and the location of the mutations are depicted. The SNP in *fgt2-1* causes an aa exchange at position 44 (L to F). Colored bars, exons; black lines, introns. Cys (C) residues predicted to be palmitoylated are indicated. (b,c) The *fgt2-1* mutant phenotype is complemented by a *pFGT2::FGT2-vYFP* construct (R331_1 and R331_2 are independent transgenic lines) at the (b) LUC expression and (c) physiological levels (cf. Figure S3e). Assays on the indicated genotypes were performed as described in Figure 1.

pFGT2::FGT2-vYFP and *pFGT2::FGT2*, respectively, at the level of LUC activity and seedling survival (Figure 2b,c; Figure S3e). *FGT2* transcript levels did not respond to HS (Figure S4a); however, regulation at the post-translational level currently cannot be excluded. In addition, overexpression of *FGT2* under control of the CaMV 35S promoter enhanced the physiological HS memory (Figure S5). In summary, several lines of evidence confirm that the phosphatase APD9/P2C79 is FGT2.

FGT2 is localized to the plasma membrane by the N-terminal domain

To investigate the function of FGT2, we studied its subcellular localization in transiently transformed *Nicotiana*

benthamiana leaves and stably transformed *A. thaliana* seedlings. In *A. thaliana*, FGT2 was restricted to the PM, where it co-localized with the PM marker NPSN12 (Geldner *et al.*, 2009) (Figure 3a). Experimental induction of plasmolysis indicated that FGT2 was not associated with the cell wall, as co-localization with the PM reporter remained after plasmolysis (Figure 3a, bottom panel). Moreover, biochemical fractionation and immunoblotting detected FGT2 mainly in the PM-enriched (microsomal) fraction, and not in the soluble fraction (Figure 3b). As the first 20 aa of the protein were annotated as a putative signal peptide (SP), we investigated their role in the targeting of FGT2 to the PM. Deletion of the putative SP (aa 2–21, *35S::FGT2*$^{\Delta(2\text{-}21)}$*-vYFP*) re-localized FGT2 to the nucleus and cytoplasm (Figures 3c and S4b,c). Recruitment to the PM may also be mediated by lipid anchors such as S-palmitoylation of cysteine residues (Chamberlain *et al.*, 2013). FGT2 harbors two predicted palmitoylation sites at the residues cysteine-12 and cysteine-152 (Ren *et al.*, 2008). Site-directed mutagenesis revealed that exchanging cysteine for glycine at position 12, but not at position 152, impaired the localization of the protein to the PM, similarly as deletion of the SP (Figure 3c). This indicates that palmitoylation of cysteine-12 may be responsible for the PM localization of FGT2.

To test whether PM localization is essential for the function of FGT2, we analyzed whether the N-terminally truncated FGT2 protein was able to complement the *fgt2-1* mutant. Constitutive expression of *FGT2*$^{\Delta(2\text{-}21)}$*-vYFP* (R332) in the *fgt2-1* background restored HS memory as demonstrated at the level of LUC activity and seedling survival (Figures 3d,e and S4d). This suggests that the SP and/or cysteine-12 palmitoylation mediate PM localization of FGT2 via lipid anchoring, and that FGT2 functions inside the cell and does not need to be attached directly to the PM to be functional.

FGT2 interacts with a phospholipase D

To analyze the molecular function and potential targets of FGT2, we identified interacting proteins using a co-immunoprecipitation (Co-IP)–liquid chromatography/mass spectrometry (LC-MS/MS) approach. To this end, native FGT2-vYFP complexes were isolated from *35S::FGT2-vYFP* plants in the parental and *fgt2-2* backgrounds, and processed for LC-MS/MS. A list of peptides was obtained that co-immunoprecipitated with FGT2-vYFP, but not with vYFP or a non-transgenic control. We identified PLDα2 (*At1g52570*) as a potential interaction partner of FGT2, as PLDα2 was present in all Co-IPs at all time points with a similar number of peptides as FGT2 (Table S1). Phospholipases of group D hydrolyze membrane lipids (Fan *et al.*, 1999; Bargmann and Munnik, 2006; Hong *et al.*, 2016), and the activity of PLDs has been implicated in plant HS responses (Mishkind *et al.*, 2009; Hong *et al.*, 2016). The fusion of PLDα2 to YFP indicated a cytoplasmic localization pattern, similar to the N-terminally truncated FGT2 (Figure 4a). This is in line with previous reports that PLDα is enriched in the soluble fraction, and is also present in the membrane fraction (Fan *et al.*, 1999). Thus, both proteins may interact in the cytoplasm or at the cytosol–PM interface.

To confirm the interaction of FGT2 and PLDα2, we performed ratiometric bimolecular fluorescence complementation (rBiFC) assays in tobacco leaves (Grefen and Blatt, 2012; Hecker *et al.*, 2015). Reconstitution of the YFP signal along the PM was observed when FGT2-nYFP and cYFP-PLDα2 were co-expressed, but not when either unfused nYFP or cYFP was (co)-expressed with the respective fusion protein (Figure 4b). We also tested whether the localization of FGT2 to the PM was required for the interaction with PLDα2. FGT2-nYFP lacking the SP (FGT2$^{\Delta(2\text{-}21)}$) still interacted with cYFP-PLDα2; however, the signal was now present in the cytoplasm. We also confirmed this interaction in stably transformed *A. thaliana* by Co-IP of PLD with FGT2$^{\Delta(2\text{-}21)}$-YFP using an antibody against the endogenous PLDα (Figure 4c). PLDα was co-purified from *35S::FGT2*$^{\Delta(2\text{-}21)}$-YFP, but not from *35S::YFP* plants after ACC and a 1 h recovery. In summary, FGT2 and PLDα2 interact independently of the subcellular localization of FGT2, which is in agreement with the ability of FGT2$^{\Delta(2\text{-}21)}$ to complement the *fgt2-1* mutant phenotype (Figure 3d,e).

PLDα2 is specifically required for HS memory and interacts with FGT2 genetically

Given the interaction of PLDα2 and FGT2 *in planta*, we next asked whether *pldα2* mutants also displayed a deficiency in HS memory. We isolated mutants for the three genes of the PLDα group, *pldα1, pldα2*, and *pldα3* (Bargmann and Munnik, 2006), and tested their HS memory. *Pldα2* and *pldα3* mutants had a reduced HS memory, while *pldα1* mutants did not differ from Col-0 (Figure S6a,b). All three *pldα* mutants were able to acquire thermotolerance as wild-type plants (Figure S7a), and showed only a minor reduction in basal thermotolerance (Figure S7b), which was unlikely to account for the observed defect in HS memory. Neither of the *pldα* mutants displayed any additional morphological phenotypes. To further test the interaction of *PLDα2* and *FGT2*, we analyzed HS memory in the *fgt2-1 pldα2* double mutant. We did not observe additivity in the HS memory assay; instead, two independently isolated double mutant lines behaved similar to the *fgt2-1* mutant (Figure 5a,b). Thus, *FGT2* and *PLDα2* both mediate HS memory and act in a common genetic pathway.

DISCUSSION

We have shown that FGT2 and PLDα2 are required for HS memory and that both proteins act in a common genetic pathway. FGT2 is the first protein involved in protein (de-)

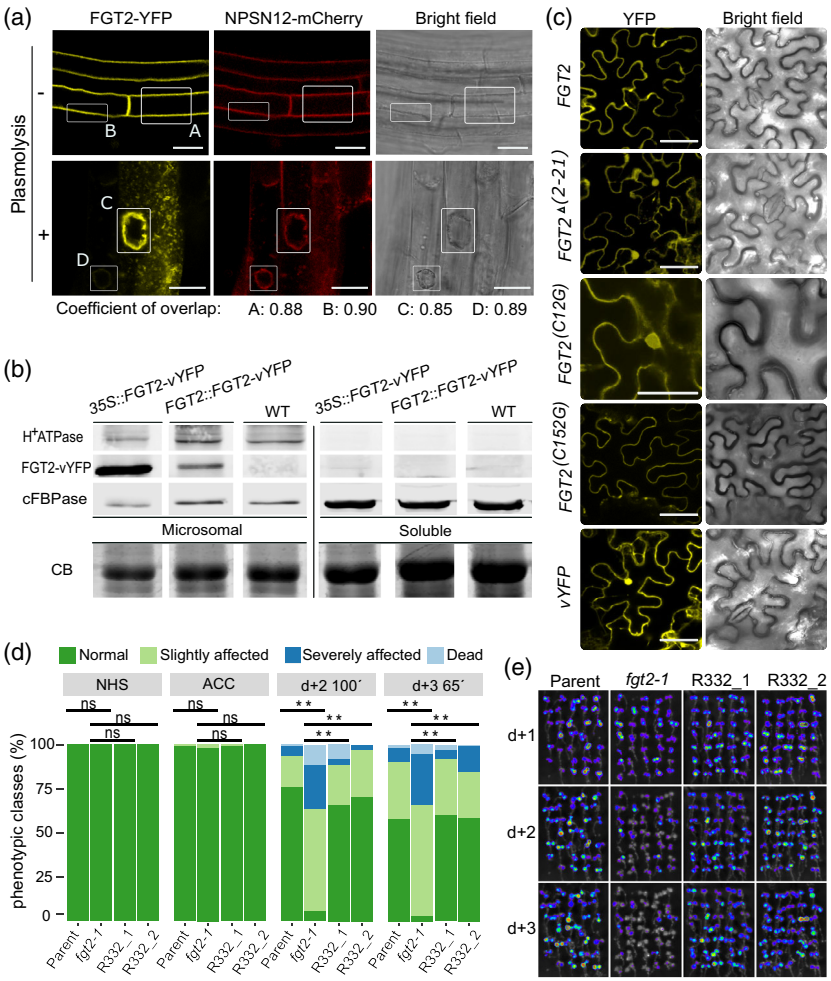

Figure 3. FGT2 localizes to the plasma membrane and functions intracellularly.
(a) FGT2 co-localizes with the PM reporter NPSN12. Five-day-old seedlings carrying *35S::FGT2-vYFP* and *pUBQ10::NPSN12-mCherry* were incubated in either water (–) or 0.8 M mannitol (+, plasmolysis) for 2 h. Root cells were imaged and the coefficient of overlap between the vYFP and mCherry signals was calculated for the indicated regions. Scale bar, 20 μm. (b) FGT2-vYFP is enriched in the microsomal (membrane) fraction. Protein extracts from transgenic lines expressing *35S::FGT2-vYFP* or *pFGT2::FGT2-vYFP* were separated into membrane-enriched and soluble fractions. Immunoblot analysis with markers for the PM (H^+-ATPase) and cytoplasm (cFBPase) was performed to confirm fractionation efficiency. CB, Coomassie Blue staining. (c) The FGT2 N-terminus (aa 2–21) is required for PM localization, as shown by transient transformation of *N. benthamiana* leaves of FGT2-*vYFP* mutated variants: *FGT2*, $FGT2^{\Delta(2\text{-}21)}$, $FGT2^{(C12G)}$, and $FGT2^{(C152G)}$. Mutation of C12, but not C152, disrupts PM localization. Scale bar, 50 μm. (d,e) Constitutive expression of $FGT2^{\Delta(2\text{-}21)}$-*vYFP* (R332_1 and R332_2 are independent transgenic lines) in the *fgt2-1* background restores (d) HS memory (cf. Figure S4c) and (e) LUC expression. Four-day-old seedlings of the indicated genotypes were treated and analyzed as in Figure 1. $^{**}P < 0.001$, Fisher's exact test.

phosphorylation to be implicated in HS memory. FGT2 is specifically required for HS memory, while immediate HS responses such as basal thermotolerance and acquisition of thermotolerance are not affected and slightly improve compared to the control line, respectively. FGT2 is a PP2C phosphatase of the D clade; other PP2C proteins have been implicated in various stress responses, most notably the A clade members in the signal transduction of the phytohormone ABA (Fuchs *et al.*, 2013). Clade D remains poorly characterized; so far, one of the nine members, APD1, has been implicated in cell expansion by regulating the activity of the PM-localized H^+-ATPase, which in turn mediates acid growth (Spartz *et al.*, 2014). Consistent with previous reports (Tovar-Mendez *et al.*, 2014), FGT2 is localized at the PM. We found that the first 21 aa are required for localization to the PM and that membrane anchoring is not crucial for FGT2 function. Thus, it appears likely that FGT2 acts as a peripheral membrane protein that is attached to the PM through lipid anchoring (Hemsley and Grierson, 2008; Aicart-Ramos *et al.*, 2011), such as palmitoylation of cysteine-12. Membrane anchoring through palmitoylation was previously described for the PP2Cs POLTERGEIST and POLTERGEIST-LIKE (Gagne and Clark, 2010).

We identified PLDα2 as an *in vivo* interaction partner of FGT2. PLD proteins hydrolyze structural phospholipids of the phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl glycerol classes, thus producing PA and the corresponding head group (Pappan *et al.*, 1998; Hong *et al.*, 2016). PLDs function in membrane degradation, vesicular transport, membrane tethering, and signal transduction through recruitment and regulation of target gene activity (Bargmann and Munnik, 2006; Hong *et al.*, 2016). This is often mediated by PA, which acts as a signaling molecule and associates with many proteins (Hou *et al.*, 2016). PLDs have been implicated in plant responses to abiotic and biotic stresses (Bargmann and Munnik, 2006; Hong *et al.*, 2016). PLDα and PLDδ act in ABA signaling by producing PA, which binds to the PP2C phosphatase ABI1 and prevents its translocation into the nucleus (Zhang *et al.*, 2004). PLD activity increases dramatically after HS (Mishkind *et al.*, 2009), but so far this could not be linked to any specific PLD gene. In tomato, PA levels increase

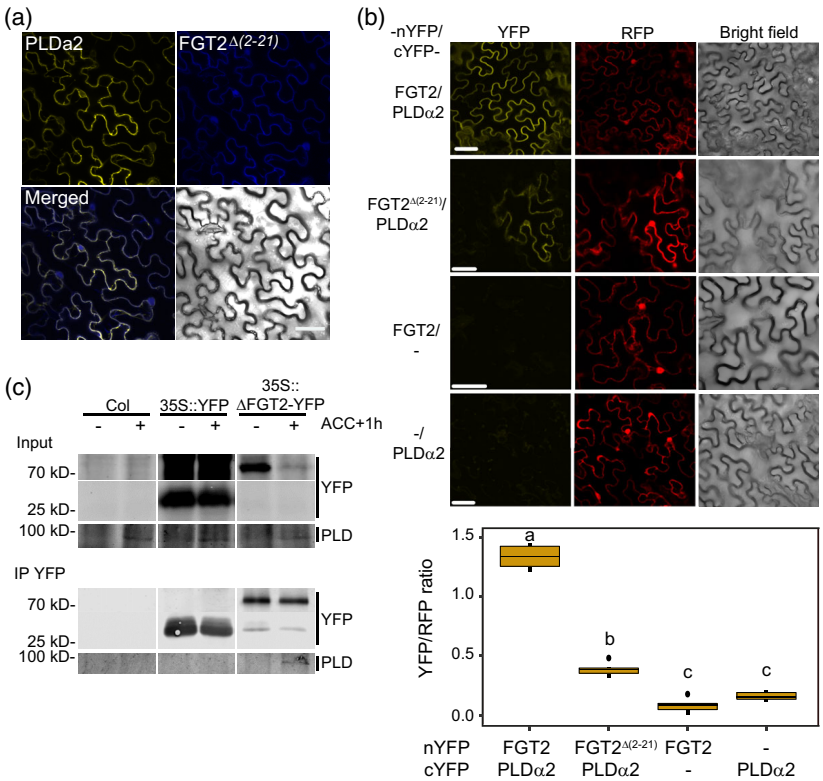

Figure 4. FGT2 interacts with phospholipase D α2 (PLDα2).
(a) *PLDα2-YFP* was transiently co-expressed in *N. benthamiana* leaves with *FGT2*$^{\Delta(2\text{-}21)}$*-turquoise*. PLDα2 is cytoplasmic but excluded from the nucleus compared to *FGT2*$^{\Delta(2\text{-}21)}$. Scale bar, 50 μm.
(b) FGT2 and PLDα2 interact in *N. benthamiana*, as shown by the rBiFC assay. Quantification of the YFP/RFP fluorescence ratio in the rBiFC assays was based on six images from three different transformed plants per combination. $P < 0.05$, LSD-test.
(c) FGT2$^{\Delta(2\text{-}21)}$ and PLD interact in A. *thaliana*, as shown by co-immunoprecipitation of FGT2$^{\Delta(2\text{-}21)}$. Protein extracts of the indicated genotypes from heat-treated (+, ACC + 1 h recovery) or control (−) samples were immunoprecipitated with anti-GFP. Proteins were detected by immunoblotting with antibodies against GFP and PLDα.

after HS and PA continues to accumulate for at least 6 h into the recovery phase (Abd-El-Haliem *et al.*, 2012). In contrast, the phospholipase C (PLC) members *AtPLC3* and *AtPLC9* are required for basal and acquired thermotolerance (Zheng *et al.*, 2012; Gao *et al.*, 2014). PLCs hydrolyze phosphatidylinositol polyphosphates, thus generating diacylglycerol and inositol-1,4,5-trisphosphate (Zheng *et al.*, 2012). Here, we found that *PLDα2* and *PLDα3* are required for HS memory, but not for acquired or basal thermotolerance. PLDα2 is a soluble protein that interacts with FGT2 at the PM, suggesting that FGT2 supports recruitment of PLDα2 to the PM. The double mutant did not show an enhanced HS memory phenotype compared to either single mutant, corroborating the idea that both proteins act in a common pathway. An interesting question for future studies is whether FGT2 dephosphorylates PLDα2 to regulate its activity in a possibly HS-dependent manner. Both proteins can be phosphorylated *in vivo*, as shown by phosphoproteomic analysis (Durek *et al.*, 2010). Alternatively, PLDα2-derived PA may regulate the activity of FGT2. It also remains to be investigated whether FGT2 also interacts with PLDα1 and PLDα3, which may be likely, given that PLDα3 is also required for HS memory.

Lipid composition changes rapidly after HS to counteract the increased membrane fluidity upon a temperature increase (Sinensky, 1974; Martiniere *et al.*, 2011). As temperature rises, saturation levels of glycolipids increase (Burgos *et al.*, 2011; Hemme *et al.*, 2014; Higashi *et al.*, 2015; Legeret *et al.*, 2016). These changes occur rapidly and have been proposed to trigger downstream signaling cascades through Ca^{2+} and ROS in HS sensing (Volkov *et al.*, 2006; Konigshofer *et al.*, 2008; Mittler *et al.*, 2012; Finka *et al.*, 2012; Rütgers *et al.*, 2017). How long they are maintained during recovery after HS is unknown. Our work suggests a model where FGT2 and PLDα2 modulate HS memory, likely through the modification of membrane lipids or PA-dependent signaling (Figure 6). PLDα2 activity may be regulated by FGT2-dependent protein dephosphorylation, which may in turn be modulated by HS. Further clarification of the pathway and the role of protein dephosphorylation mediated by FGT2 remains a challenge for future studies.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

The genetic background was *A. thaliana* Col-0 (except for *fgt2-3* analysis, which was performed in a RATM line in a No-0 background and which was compared to the corresponding parental lines DS and AC (Kuromori *et al.*, 2004)). *Hsfa2-1* (Charng *et al.*, 2006), *hsp101* (Stief *et al.*, 2014), *pUBQ10::NPSN12-mCherry* (wave_131R) (Geldner *et al.*, 2009), and *pHSA32::HSA32-LUC* (Brzezinka *et al.*, 2016) were previously described. *Fgt2-2* (SALK_118712) was obtained from Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK) and backcrossed twice before

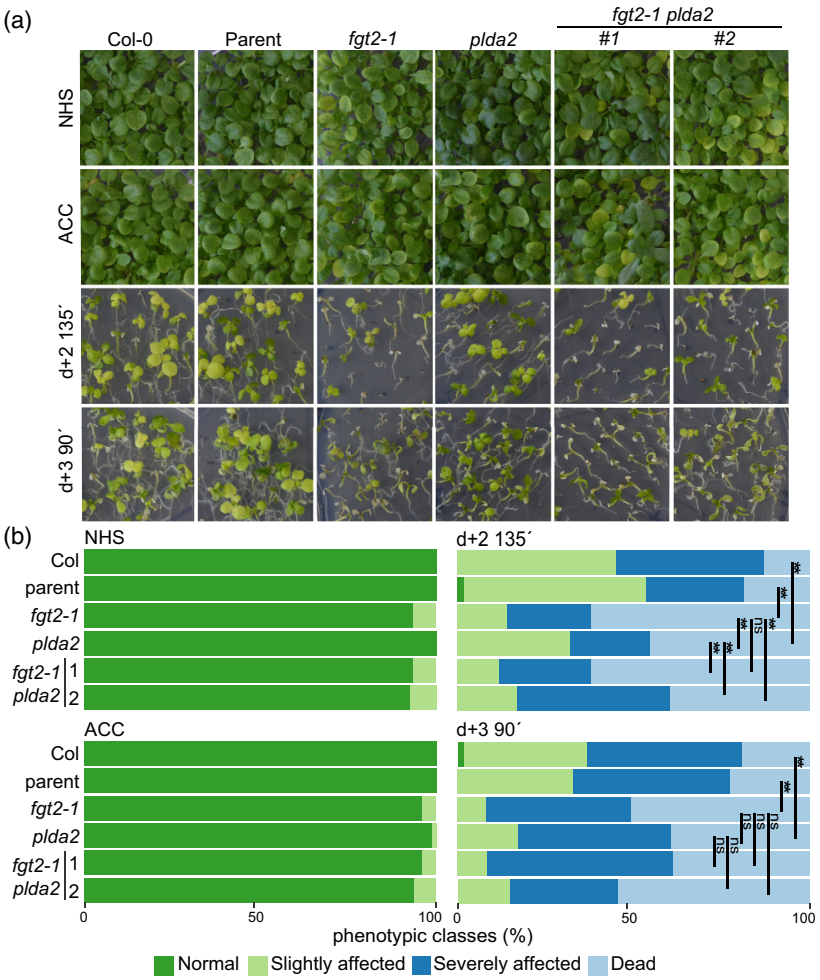

Figure 5. Phospholipase D α2 (PLDα2) and FGT2 interact genetically and are together required for HS memory.
HS memory in the *fgt2-1 pldα2* double mutant is similar to that in the *fgt2-1* single mutant. Four-day-old seedlings of the indicated genotypes were treated and analyzed as in Figure 1. (a) Representative seedling images. (b) Quantification as described in Figure 1b. $*P < 0.05$; $**P < 0.001$, Fisher's exact test.

analysis. *Fgt2-3* (pst16006) was obtained from RIKEN, Wako, Japan. DS, AC (Kuromori *et al.*, 2004), *pldα1* (GK_332D11), *pldα2* (Salk_098375C), and *pldα3* (Salk_009648) were obtained from NASC. Surface-sterilized seeds were grown on GM medium with 1% (w/v) glucose under 16 h/8 h light/dark cycles at 23°C/21°C. Details on transgene constructions can be found in the Supplementary Materials and Methods, sequences of oligonucleotides can be found in Table S2.

HS physiological assays and LUC reporter assay

HS assays were performed on 4-day-old seedlings as described (Brzezinka *et al.*, 2016). Basal thermotolerance was tested by direct exposure to 44°C for 10–50 min; acquisition of thermotolerance was tested by exposure to 37°C for 1 h, followed by recovery at 22°C for 90 min and HS at 44°C for 120–200 min; HS memory was tested by applying the acclimating HS treatment (ACC, 37°C for 1 h, 22°C for 90 min, and 44°C for 45 min) and 2–3 days later a tester HS at 44°C for 65–140 min. To assay better HS memory, a shortened priming treatment (37°C for 1 h) was used (cf. Figure S5). After treatment, seedlings were returned to normal growth conditions and analyzed 14 days later. For each time point, three technical replicates were done (~84 individuals per genotype) and the whole experiment was repeated at least twice independently. All genotypes of one assay were grown on the same plate.

Subcellular localization analysis

Five-day-old seedlings were incubated in 0.8 M mannitol for 2 h to induce plasmolysis and imaged as described below. For biochemical fractionation 2 g of 5-day-old seedlings were ground in liquid nitrogen and resuspended in 5 ml extraction buffer (20 mM Tris-HCl pH 8.8, 150 mM NaCl, 1 mM EDTA, 20 % glycerol, 1 mM PMSF, protease inhibitor cocktail [Roche, Basel, Switzerland]). Cell debris was removed at 16 000 ***g*** for 20 min at 4°C and the supernatant was transferred into a new tube and subjected to ultracentrifugation at 100 000 ***g*** for 1.5 h at 4°C. The soluble fraction was kept, and the microsomal fraction was resuspended in dissolving buffer (1% Triton X-100, 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM PMSF, protease inhibitor cocktail [Roche]) and sonicated with a Diagenode (Seraing, Belgium) Bioruptor (two cycles of 30 sec on/off) on low-intensity settings. For SDS-PAGE, equal amounts of protein extracts from both fractions were denatured for 1 h with 8 M urea in loading buffer. For immunoblotting, antibodies against H^+-ATPase (AS07260), cFBPase (AS04043), PLDα (AS122364, all Agrisera, Vännäs, Sweden), and GFP (ab290, Abcam, Cambridge, UK) were used.

Transient expression in tobacco

Five- to seven-week-old *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* strain GV2260 containing the

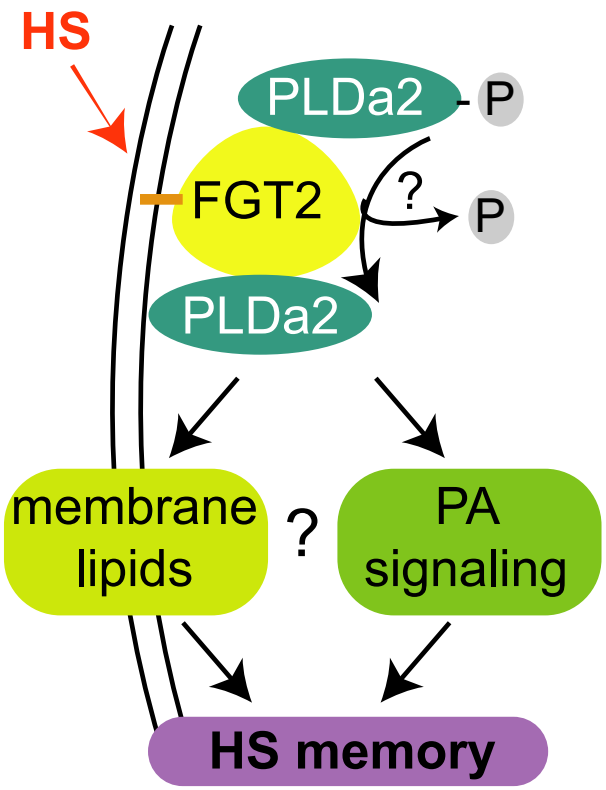

Figure 6. FGT2 and PLDα2 modulate lipid dynamics and play a role in HS memory.
FGT2 is a PM-anchored protein phosphatase that interacts with PLDα2 and may regulate its phosphorylation status. PLDα2 activity produces the signaling molecule PA and modulates membrane lipid metabolism. HS memory may be modulated by PA signaling or membrane lipid dynamics.

respective constructs as described (Sparkes *et al.*, 2006). Analysis was performed 2–3 days after infiltration. Relative signal intensity of the fluorophores was quantified in ImageJ (https://imagej.net/ImageJ).

Immunoprecipitation and mass spectrometry

Five-day-old seedlings (1.5 g) were subjected to the indicated treatments and plant material was frozen in liquid nitrogen and ground. Extracts were resuspended in extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2% [v/v] Triton X-100, protease inhibitor cocktail [Roche]), cleaned from cell debris by centrifugation, and incubated with α-GFP beads (Miltenyi, Bergisch Gladbach, Germany) for 1.5 h at 4°C. Protein complexes were isolated using the Miltenyi Biotec kit as described in (Smaczniak *et al.*, 2012) with the following modifications: An additional wash with 20 mM Tris-HCl was included and the protein complexes were eluted with 8 M urea. Sample preparation for LC-MS/MS analysis and peptide detection and analysis using MaxQuant was performed as described in (Brzezinka *et al.*, 2016). To identify potential FGT2 interactors, peptide numbers were subjected to hierarchical cluster analysis using Euclidian distances and complete linkage using Perseus 1.5.8.5 (Tyanova *et al.*, 2016).

Gene expression analyses

RNA extraction, reverse transcription, and qPCR were performed as described (Stief *et al.*, 2014; Lämke *et al.*, 2016). Expression values were normalized to the reference *At4g26410* (Czechowski *et al.*, 2005). Expression of *pHSA32::HSA32-LUC* was determined in different genetic backgrounds grown on the same plate as previously described (Brzezinka *et al.*, 2016).

Construction of transgenic lines

For *FGT2::FGT2-vYFP* (R331), a genomic fragment including the FGT2 coding sequence and the 1344 bp promoter sequence was amplified with primers 1386/1387. A second fragment 625 bp downstream of the *FGT2* stop codon was amplified with 1296/1297 primers. Both fragments were cloned into pIB21 via *Sal*I/*Bam*HI and *Bgl*II/*Eco*RI, respectively, generating a *FGT2-vYFP* in-frame fusion. The fragment was transferred into *pBarMAP* (IB20) via *Asc*I/*Pac*I. For *35S::FGT2* (SA19), primers 1096/1098 were used to amplify *FGT2* and the fragment was inserted into pBarM (IB485) via *Bam*HI. For *35S::FGT2-vYFP* (SA18) a genomic fragment encompassing the *FGT2* sequence was amplified with primers 1096/1097 and cloned into *pBarM* (IB30) via *Bam*HI. For *35S::FGT2*$^{\Delta(2\text{-}21)}$*-vYFP* (R332), a genomic fragment lacking the sequence encoding the first 21 aa of *FGT2* was amplified with primers 1271/1272. This fragment was cloned into *pBarM* (IB30) via *BamH*I.

All constructs were transformed into *A. tumefaciens* strain GV3101 and stably transformed into *A. thaliana* by floral dip.

For transient protein localization studies of FGT2 with tagged vYFP, truncated and mutant versions were amplified from genomic DNA. The fragments were cloned into pBarM (IB30) via *Bam*HI sites to insert the transgene between the 35S promoter and vYFP tag. The *35S::FGT2*$^{\Delta(2\text{-}21)}$*-vYFP* construct was described above. For *35S::FGT2*$^{(C12G)}$*-vYFP,* primers 1421/1425 were used (1421 harbors the mutation). In the case of *35S::FGT2*$^{(C152G)}$*-vYFP,* two genomic fragments up- and downstream of the mutation site were amplified with primers 1422/1423 and 1424/1425, respectively. Then, both fragments were combined and used for fusion PCR with primers 1422/1425 to generate the complete *FGT2*$^{(C152G)}$. For protein–protein interaction studies *in planta* using the rBiFC system (Grefen and Blatt, 2012), FGT2, PLDα2, and APD2 were amplified from cDNA, using the primer combinations 2744/2745, 2746/2748, and 2749/2750, respectively. Subsequently, Gateway cloning (Invitrogen, Waltham, MA, USA) was used to generate the 2-in-1 constructs as described (Hecker *et al.*, 2015). For PLDα2 localization, cDNAs were cloned into pFRET-gc-2in1-CN from the same vector collection to yield pFRET-gc-2in1-CN_FGT2$^{\Delta 2\text{-}21}$/PLDα2.

Microscopy

Confocal laser scanning microscopy was performed using a Zeiss LSM710 (Zeiss, Jena, Germany). Plant material was mounted in ddH_2O and visualized using a C-Apochromat 40×/1.20 W objective. vYFP and mCherry/RFP were excited with a 514-nm argon laser (emission 515–575 nm) and a 561-nm solid-state laser (emission 608–659), respectively. For co-localization and rBiFC assays, both fluorophores were detected in separate tracks to reduce bleed-through. All images were captured under the same settings to allow comparability. The coefficient of overlap (Figure 3a) was calculated using JACoP in ImageJ (Bolte and Cordelieres, 2006).

ACCESSION NUMBERS

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers

At5g66080 (*FGT2*), *At4g21320* (*HSA32*), *At2g26150* (*HSFA2*), *At1g48240* (*NPSN12*), *At3g15730* (*PLDα1*), *At1g52570* (*PLDα2*), and *At5g25370* (*PLDα3*).

ACKNOWLEDGMENTS

We acknowledge NASC and RIKEN for seeds, C. Kappel for bioinformatics support, F. Jähne, S. Wenk, and E. Benke for technical assistance, and C. Schmidt and D. Mäker for excellent plant care. We also thank M. Lenhard, A. Sicard, and members of our laboratory for helpful comments. IB acknowledges support from an ERC CoG (725295), a Sofja-Kovalevskaja-Award (Alexander-von-Humboldt-Foundation), and CRC 973 Project A2 (DFG). RUC was supported by scholarships from the CRC 973, the Potsdam Graduate School, and the IMPRS-PMPG program of the Max-Planck-Society. Open access funding enabled and organized by ProjektDEAL.

AUTHOR CONTRIBUTIONS

RUC, TF, NP, SA, and IB designed the research; RUC, TF, NP, SA, KB, MG, and AG performed the research; RUC, TF, NP, SA, KB, MG, AG, and IB analyzed the data; and RUC and IB wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Characterization of HS responses and HS memory in *fgt2-1*.

Figure S2. Protein alignments of FGT2 with related proteins from *A. thaliana* and other plant species.

Figure S3. Characterization of HS responses in additional *fgt2* alleles and transgenic complementation of *fgt2-1*.

Figure S4. Expression of *FGT2* and characterization of the N-terminally truncated $FGT2^{\Delta(2\text{-}21)}$.

Figure S5. Overexpression of *FGT2* enhances HS memory.

Figure S6. Characterization of HS memory in *pldα* mutants.

Figure S7. Characterization of HS responses in *pldα* mutants.

Table S1. PLDα2 co-immunoprecipitates with FGT2-vYFP, as detected by Co-IP–LC-MS/MS.

Table S2. List of oligonucleotides used in this study.

REFERENCES

Abd-El-Haliem, A., Meijer, H.J., Tameling, W.I., Vossen, J.H. and Joosten, M.H. (2012) Defense activation triggers differential expression of phospholipase-C (PLC) genes and elevated temperature induces phosphatidic acid (PA) accumulation in tomato. *Plant Signal. Behav.* **7**, 1073–1078.

Aicart-Ramos, C., Valero, R.A. and Rodriguez-Crespo, I. (2011) Protein palmitoylation and subcellular trafficking. *Biochem. Biophys. Acta*, **1808**, 2981–2994.

Bargmann, B.O. and Munnik, T. (2006) The role of phospholipase D in plant stress responses. *Curr. Opin. Plant Biol.* **9**, 515–522.

Battisti, D.S. and Naylor, R.L. (2009) Historical warnings of future food insecurity with unprecedented seasonal heat. *Science*, **323**, 240–244.

Bäurle, I. (2016) Plant heat adaptation: priming in response to heat stress. *F1000Research*, **5**, 694.

Bolte, S. and Cordelieres, F.P. (2006) A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc.* **224**, 213–232.

Brzezinka, K., Altmann, S., Czesnick, H. *et al.* (2016) Arabidopsis FORGETTER1 mediates stress-induced chromatin memory through nucleosome remodeling. *eLife*, **5**, e17061.

Burgos, A., Szymanski, J., Seiwert, B., Degenkolbe, T., Hannah, M.A., Giavalisco, P. and Willmitzer, L. (2011) Analysis of short-term changes in the Arabidopsis thaliana glycerolipidome in response to temperature and light. *Plant J.* **66**, 656–668.

Chamberlain, L.H., Lemonidis, K., Sanchez-Perez, M., Werno, M.W., Gorleku, O.A. and Greaves, J. (2013) Palmitoylation and the trafficking of peripheral membrane proteins. *Biochem. Soc. Trans.* **41**, 62–66.

Charng, Y.Y., Liu, H.C., Liu, N.Y., Hsu, F.C. and Ko, S.S. (2006) Arabidopsis Hsa32, a novel heat shock protein, is essential for acquired thermotolerance during long recovery after acclimation. *Plant Physiol.* **140**, 1297–1305.

Charng, Y.Y., Liu, H.C., Liu, N.Y., Chi, W.T., Wang, C.N., Chang, S.H. and Wang, T.T. (2007) A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. *Plant Physiol.* **143**, 251–262.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. and Scheible, W.R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol.* **139**, 5–17.

Ding, Y., Fromm, M. and Avramova, Z. (2012) Multiple exposures to drought 'train' transcriptional responses in Arabidopsis. *Nat. Commun.* **3**, 740.

Durek, P., Schmidt, R., Heazlewood, J.L., Jones, A., MacLean, D., Nagel, A., Kersten, B. and Schulze, W.X. (2010) PhosPhAt: the Arabidopsis thaliana phosphorylation site database. An update. *Nucleic Acids Res.* **38**, D828–D834.

Fan, L., Zheng, S., Cui, D. and Wang, X. (1999) Subcellular distribution and tissue expression of phospholipase Dalpha, Dbeta, and Dgamma in Arabidopsis. *Plant Physiol.* **119**, 1371–1378.

Feng, X.J., Li, J.R., Qi, S.L., Lin, Q.F., Jin, J.B. and Hua, X.J. (2016) Light affects salt stress-induced transcriptional memory of P5CS1 in Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **113**, E8335–E8343.

Finka, A., Cuendet, A.F., Maathuis, F.J., Saidi, Y. and Goloubinoff, P. (2012) Plasma membrane cyclic nucleotide gated calcium channels control land plant thermal sensing and acquired thermotolerance. *Plant Cell*, **24**, 3333–3348.

Fuchs, S., Grill, E., Meskiene, I. and Schweighofer, A. (2013) Type 2C protein phosphatases in plants. *FEBS J.* **280**, 681–693.

Gagne, J.M. and Clark, S.E. (2010) The Arabidopsis stem cell factor POLTERGEIST is membrane localized and phospholipid stimulated. *Plant Cell*, **22**, 729–743.

Gao, K., Liu, Y.L., Li, B., Zhou, R.G., Sun, D.Y. and Zheng, S.Z. (2014) Arabidopsis thaliana phosphoinositide-specific phospholipase C isoform 3 (AtPLC3) and AtPLC9 have an additive effect on thermotolerance. *Plant Cell Physiol.* **55**, 1873–1883.

Geldner, N., Denervaud-Tendon, V., Hyman, D.L., Mayer, U., Stierhof, Y.D. and Chory, J. (2009) Rapid, combinatorial analysis of membrane compartments in intact plants with a multicolor marker set. *Plant J.* **59**, 169–178.

Grefen, C. and Blatt, M.R. (2012) A 2in1 cloning system enables ratiometric bimolecular fluorescence complementation (rBiFC). *Biotechniques*, **53**, 311–314.

Hecker, A., Wallmeroth, N., Peter, S., Blatt, M.R., Harter, K. and Grefen, C. (2015) Binary 2in1 Vectors Improve in Planta (Co)localization and Dynamic Protein Interaction Studies. *Plant Physiol.* **168**, 776–787.

Hemme, D., Veyel, D., Muhlhaus, T. *et al.* (2014) Systems-wide analysis of acclimation responses to long-term heat stress and recovery in the photosynthetic model organism Chlamydomonas reinhardtii. *Plant Cell*, **26**, 4270–4297.

Hemsley, P.A. and Grierson, C.S. (2008) Multiple roles for protein palmitoylation in plants. *Trends Plant Sci.* **13**, 295–302.

Higashi, Y., Okazaki, Y., Myouga, F., Shinozaki, K. and Saito, K. (2015) Landscape of the lipidome and transcriptome under heat stress in Arabidopsis thaliana. *Sci. Rep.* **5**, 10533.

Hilker, M., Schwachtje, J., Baier, M. *et al.* (2016) Priming and memory of stress responses in organisms lacking a nervous system. *Biol. Rev. Camb. Philos. Soc.* **91**, 1118–1133.

Hong, Y., Zhao, J., Guo, L., Kim, S.C., Deng, X., Wang, G., Zhang, G., Li, M. and Wang, X. (2016) Plant phospholipases D and C and their diverse functions in stress responses. *Prog. Lipid Res.* **62**, 55–74.

Hou, Q., Ufer, G. and Bartels, D. (2016) Lipid signalling in plant responses to abiotic stress. *Plant Cell Environ.* **39**, 1029–1048.

Jaskiewicz, M., Conrath, U. and Peterhänsel, C. (2011) Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* **12**, 50–55.

Konigshofer, H., Tromballa, H.W. and Loppert, H.G. (2008) Early events in signalling high-temperature stress in tobacco BY2 cells involve alterations in membrane fluidity and enhanced hydrogen peroxide production. *Plant Cell Environ.* **31**, 1771–1780.

Kuromori, T., Hirayama, T., Kiyosue, Y., Takabe, H., Mizukado, S., Sakurai, T., Akiyama, K., Kamiya, A., Ito, T. and Shinozaki, K. (2004) A collection of 11 800 single-copy Ds transposon insertion lines in Arabidopsis. *Plant J.* **37**, 897–905.

Lämke, J. and Bäurle, I. (2017) Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. *Genome Biol.* **18**, 124.

Lämke, J., Brzezinka, K., Altmann, S. and Bäurle, I. (2016) A hit-and-run heat shock factor governs sustained histone methylation and transcriptional stress memory. *EMBO J.* **35**, 162–175.

Legeret, B., Schulz-Raffelt, M., Nguyen, H.M., Auroy, P., Beisson, F., Peltier, G., Blanc, G. and Li-Beisson, Y. (2016) Lipidomic and transcriptomic analyses of Chlamydomonas reinhardtii under heat stress unveil a direct route for the conversion of membrane lipids into storage lipids. *Plant Cell Environ.* **39**, 834–847.

Liu, H.C. and Charng, Y.Y. (2013) Common and distinct functions of Arabidopsis class A1 and A2 heat shock factors in diverse abiotic stress responses and development. *Plant Physiol.* **163**, 276–290.

Liu, H.C., Liao, H.T. and Charng, Y.Y. (2011) The role of class A1 heat shock factors (HSFA1s) in response to heat and other stresses in Arabidopsis. *Plant Cell Environ.* **34**, 738–751.

Lobell, D.B., Schlenker, W. and Costa-Roberts, J. (2011) Climate trends and global crop production since 1980. *Science*, **333**, 616–620.

Luna, E., Bruce, T.J., Roberts, M.R., Flors, V. and Ton, J. (2012) Next-generation systemic acquired resistance. *Plant Physiol.* **158**, 844–853.

Martiniere, A., Shvedunova, M., Thomson, A.J., Evans, N.H., Penfield, S., Runions, J. and McWatters, H.G. (2011) Homeostasis of plasma membrane viscosity in fluctuating temperatures. *New Phytol.* **192**, 328–337.

Mishkind, M., Vermeer, J.E., Darwish, E. and Munnik, T. (2009) Heat stress activates phospholipase D and triggers PIP accumulation at the plasma membrane and nucleus. *Plant J.* **60**, 10–21.

Mishra, S.K., Tripp, J., Winkelhaus, S., Tschiersch, B., Theres, K., Nover, L. and Scharf, K.D. (2002) In the complex family of heat stress transcription factors, HsfA1 has a unique role as master regulator of thermotolerance in tomato. *Genes Dev.* **16**, 1555–1567.

Mittler, R., Finka, A. and Goloubinoff, P. (2012) How do plants feel the heat? *Trends Biochem. Sci.* **37**, 118–125.

Moellering, E.R. and Benning, C. (2011) Galactoglycerolipid metabolism under stress: a time for remodeling. *Trends Plant Sci.* **16**, 98–107.

Nishizawa-Yokoi, A., Nosaka, R., Hayashi, H. *et al.* (2011) HsfA1d and HsfA1e involved in the transcriptional regulation of HsfA2 function as key regulators for the Hsf signaling network in response to environmental stress. *Plant Cell Physiol.* **52**, 933–945.

Ohama, N., Sato, H., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2017) Transcriptional Regulatory Network of Plant Heat Stress Response. *Trends Plant Sci.* **22**, 53–65.

Pappan, K., Austin-Brown, S., Chapman, K.D. and Wang, X. (1998) Substrate selectivities and lipid modulation of plant phospholipase D alpha, -beta, and -gamma. *Arch. Biochem. Biophys.* **353**, 131–140.

Rasmann, S., De Vos, M., Casteel, C.L., Tian, D., Halitschke, R., Sun, J.Y., Agrawal, A.A., Felton, G.W. and Jander, G. (2012) Herbivory in the previous generation primes plants for enhanced insect resistance. *Plant Physiol.* **158**, 854–863.

Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y. and Yao, X. (2008) CSS-Palm 2.0: an updated software for palmitoylation sites prediction. *Protein Eng. Des. Sel.* **21**, 639–644.

Rütgers, M., Muranaka, L.S., Schulz-Raffelt, M., Thoms, S., Schurig, J., Willmund, F. and Schroda, M. (2017) Not changes in membrane fluidity but proteotoxic stress triggers heat shock protein expression in Chlamydomonas reinhardtii. *Plant Cell Environ.* **40**, 2987–3001.

Saidi, Y., Finka, A., Muriset, M., Bromberg, Z., Weiss, Y.G., Maathuis, F.J. and Goloubinoff, P. (2009) The heat shock response in moss plants is regulated by specific calcium-permeable channels in the plasma membrane. *Plant Cell*, **21**, 2829–2843.

Sani, E., Herzyk, P., Perrella, G., Colot, V. and Amtmann, A. (2013) Hyperosmotic priming of Arabidopsis seedlings establishes a long-term somatic memory accompanied by specific changes of the epigenome. *Genome Biol.* **14**, R59.

Scharf, K.D., Berberich, T., Ebersberger, I. and Nover, L. (2012) The plant heat stress transcription factor (Hsf) family: structure, function and evolution. *Biochem. Biophys. Acta*, **1819**, 104–119.

Schramm, F., Ganguli, A., Kiehlmann, E., Englich, G., Walch, D. and von Koskull-Doring, P. (2006) The heat stress transcription factor HsfA2 serves as a regulatory amplifier of a subset of genes in the heat stress response in Arabidopsis. *Plant Mol. Biol.* **60**, 759–772.

Schramm, F., Larkindale, J., Kiehlmann, E., Ganguli, A., Englich, G., Vierling, E. and von Koskull-Doring, P. (2008) A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of Arabidopsis. *Plant J.* **53**, 264–274.

Schweighofer, A., Hirt, H. and Meskiene, I. (2004) Plant PP2C phosphatases: emerging functions in stress signaling. *Trends Plant Sci.* **9**, 236–243.

Sedaghatmehr, M., Mueller-Roeber, B. and Balazadeh, S. (2016) The plastid metalloprotease FtsH6 and small heat shock protein HSP21 jointly regulate thermomemory in Arabidopsis. *Nat. Commun.* **7**, 12439.

Sinensky, M. (1974) Homeoviscous adaptation–a homeostatic process that regulates the viscosity of membrane lipids in Escherichia coli. *Proc. Natl. Acad. Sci. USA*, **71**, 522–525.

Singh, P., Yekondi, S., Chen, P.W., Tsai, C.H., Yu, C.W., Wu, K. and Zimmerli, L. (2014) Environmental history modulates arabidopsis pattern-triggered immunity in a HISTONE ACETYLTRANSFERASE1-dependent manner. *Plant Cell*, **26**, 2676–2688.

Smaczniak, C., Immink, R.G., Muino, J.M. *et al.* (2012) Characterization of MADS-domain transcription factor complexes in Arabidopsis flower development. *Proc. Natl. Acad. Sci. USA*, **109**, 1560–1565.

Sparkes, I.A., Runions, J., Kearns, A. and Hawes, C. (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat. Protoc.* **1**, 2019–2025.

Spartz, A.K., Ren, H., Park, M.Y., Grandt, K.N., Lee, S.H., Murphy, A.S., Sussman, M.R., Overvoorde, P.J. and Gray, W.M. (2014) SAUR inhibition of PP2C-D phosphatases activates plasma membrane H+-ATPases to promote cell expansion in Arabidopsis. *Plant Cell*, **26**, 2129–2142.

Stief, A., Altmann, S., Hoffmann, K., Pant, B.D., Scheible, W.-R. and Bäurle, I. (2014) Arabidopsis miR156 regulates tolerance to recurring environmental stress through SPL transcription factors. *Plant Cell*, **26**, 1792–1807.

Takahashi, H., Ozawa, A., Nemoto, K., Nozawa, A., Seki, M., Shinozaki, K., Takeda, H., Endo, Y. and Sawasaki, T. (2012) Genome-wide biochemical analysis of Arabidopsis protein phosphatase using a wheat cell-free system. *FEBS Lett.* **586**, 3134–3141.

Tovar-Mendez, A., Miernyk, J.A., Hoyos, E. and Randall, D.D. (2014) A functional genomic analysis of Arabidopsis thaliana PP2C clade D. *Protoplasma*, **251**, 265–271.

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M. and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat. Methods*, **13**, 731–740.

Volkov, R.A., Panchuk, I.I., Mullineaux, P.M. and Schoffl, F. (2006) Heat stress-induced H(2)O (2) is required for effective expression of heat shock genes in Arabidopsis. *Plant Mol. Biol.* **61**, 733–746.

Wibowo, A., Becker, C., Marconi, G. *et al.* (2016) Hyperosmotic stress memory in Arabidopsis is mediated by distinct epigenetically labile sites in the genome and is restricted in the male germline by DNA glycosylase activity. *eLife*, **5**, e13546.

Wu, T.Y., Juan, Y.T., Hsu, Y.H., Wu, S.H., Liao, H.T., Fung, R.W. and Charng, Y.Y. (2013) Interplay between heat shock proteins HSP101 and HSA32 prolongs heat acclimation memory posttranscriptionally in Arabidopsis. *Plant Physiol.* **161**, 2075–2084.

Xue, T., Wang, D., Zhang, S., Ehlting, J., Ni, F., Jakab, S., Zheng, C. and Zhong, Y. (2008) Genome-wide and expression analysis of protein phosphatase 2C in rice and Arabidopsis. *BMC Genom.* **9**, 550.

Yeh, C.H., Kaplinsky, N.J., Hu, C. and Charng, Y.Y. (2012) Some like it hot, some like it warm: phenotyping to explore thermotolerance diversity. *Plant Sci.* **195**, 10–23.

Yoshida, T., Ohama, N., Nakajima, J. *et al.* (2011) Arabidopsis HsfA1 transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Mol. Genet. Genomics*, **286**, 321–332.

Zhang, W., Qin, C., Zhao, J. and Wang, X. (2004) Phospholipase D alpha 1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. *Proc. Natl. Acad. Sci. USA*, **101**, 9508–9513.

Zhang, W., Zhou, R.G., Gao, Y.J., Zheng, S.Z., Xu, P., Zhang, S.Q. and Sun, D.Y. (2009) Molecular and genetic evidence for the key role of AtCaM3 in heat-shock signal transduction in Arabidopsis. *Plant Physiol.* **149**, 1773–1784.

Zheng, S.Z., Liu, Y.L., Li, B., Shang, Z.L., Zhou, R.G. and Sun, D.Y. (2012) Phosphoinositide-specific phospholipase C9 is involved in the thermotolerance of Arabidopsis. *Plant J.* **69**, 689–700.